The breeding system of the European grayling *(Thymallus thymallus)* – a genetic perspective

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Abstract

Breeding systems for species with cryptic lives are difficult to examine in nature based on purely observational studies. In this study, a genetic approach was used to investigate the breeding system of a population of a stream spawning salmonid, the European grayling (*Thymallus thymallus*), from a tributary in the Norwegian lake system, Lake Lesjaskogsvatnet. For this purpose, highly polymorphic microsatellite loci were used to genotype adult grayling sampled in 2008. Those genotypes were in turn used to assign parentage to > 800 grayling fry sampled in 2008, which were genotyped for the same loci. In order to achieve the best possible results, I applied two different methods of parentage assignment and compared their results. Despite differences in individual results, overall, both methods were consistent in their major findings. Both confirm an apparent polygynandrous grayling breeding system, that is, that grayling of both sexes mated successfully with more than one partner during spawning in 2008 in Lake Lesjaskogsvatnet. In addition, a large variation in reproductive success and a reproductive skew for both sexes were observed. However, this variance in individual reproductive success could not conclusively be explained, neither with body length nor with the timing of spawning migration.

Preface

I would like to thank:

- My supervisor L. Asbjørn Vøllestad for giving me excellent advice and superb help during the process of writing this thesis. For always having your door open, answering questions and for being friendly and helpful.

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- My parents for encouragement and financial support throughout the years at Blindern. Linda Tveterås for her patience and support during the writing process.

Table of contents

1 Introduction

Gaining knowledge on a species' breeding system is important to be able to understand all the factors that are influencing a breeding individual's chance of reproductive success, and consequently its fitness. Thus, understanding the dynamics of a species' breeding system is vital to gain insight into possible processes of sexual selection, by looking for sex specific traits that enhance individual reproductive success. The concept of sexual selection was first introduced by Darwin (1859), and explains selection working through the competition for mating partners. Natural selection acts to enhance an individual's chance of survival and leads to adaptations like feeding advantages or anti-predatory behaviour. Sexual selection on the other hand, produces characters that aid in the competition within one sex for access to the other (intrasexual selection), as increased body size, aggressive behaviour or weapons for fighting. Or, the other form of sexual selection, intersexual selection, that increases the attractiveness of individuals of one sex to members of the other, as sexual dimorphism and various displays (Barnard 2004). This may lead to skewed individual reproductive success in a population where some individuals produce a high proportion of one season's progeny.

The term "breeding system" was defined by Reynolds (1996) as a description of the behaviour during mating, and the level of parental care carried out by two sexes. This definition includes variation in the form and extent of parental care and pair bonds, the number of mates, the degree of mate choice and the forms of courtship and mate competition. The strategies observed in nature are trade-offs between several factors, e.g. the competition intensity, the cost of gametes and parental care and degree of mate choice. Thus, a species' mating behaviour can vary from season to season and place to place, depending on the current conditions. In the animal kingdom, four types of mating systems are recognized, based on the number of breeding partners an individual has during one reproductive cycle (monogamy, polygyny, polyandry and polygynandry; Figure 1). A mating system is defined as monogamous when both males and females mate with only one partner during a reproductive cycle. This is most common in species were the new-born benefits from parental care from both parents, and monogamous species are found among birds (Quillfeldt *et al.* 2001; Barnard 2004), bony fishes (Jones *et al.* 1998; DeWoody *et al.* 2000) and mammals (Cantoni and Vogel 1989; Brotherton *et al.* 1997). However, recent genetic studies have shown that extra-pair copulations, genetic polygamy, in socially monogamous species may be common (Reynolds 1996; Rocha *et al.* 2008). Polygynous mating is when males mate with more than

one female, and females mate with only one male per cycle. This is the most common mating system observed in the animal kingdom (Barnard 2004), and polygynous species are frequently sexual dimorphic (Cooper *et al.* 2011). In polyandrous systems one female mates with more than one male, and males mate with only one female per cycle (Barnard 2004). Polyandrous species are less common, but more of them are consistently revealed, along with the increase in genetic investigations performed. Polyandry is prevalent in some bird species and pipefishes (Reynolds 1996; Zeh and Zeh 1996; Andersson 2005). Polygynandrous mating is when both males and females mate with more than one partner of the opposite sex during a reproductive cycle (Barnard 2004). Due to recent genetic studies, many previously polygynous mammalian species are now classified as polygynandrous (Munroe and Koprowski 2011). Further, sexual monomorphism or weak sexual dimorphism may be observed in polygynandrous species (Cooper *et al.* 2011).

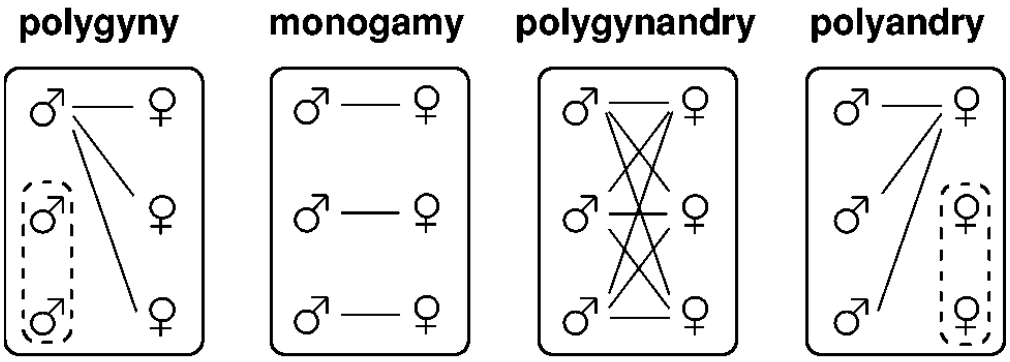

Figure 1: Illustration of the four main (genetic) mating systems found in nature. Lines between males and females represent parent pairs. Revised from Avise et al. (2002).

Among the approximately 28,000 described species of bony fish (Nelson 2006) there exists a wide variety of observed systems for breeding and parental care, from genetic monogamy to polygynandry, and from a lack of parental care to male pregnancy (Avise *et al.* 2002; Rocha *et al.* 2008). There have been numerous studies on the breeding systems of species belonging to the Salmonidae family. One of the reasons for that is probably their economical and recreational importance as well as their large variation of very different species, e.g. Atlantic salmon *Salmo salar*, coho salmon *Oncorhynchus kisutch*, pink salmon *Oncorhynchus gorbuscha*, sockeye salmon *Oncorhynchus nerka*, brown trout *Salmo trutta*, brook trout *Salvelinus fontalis* and arctic char *Salvelinus alpinus*. The typical salmonid breeding system is polygamous (Altukhov *et al.* 2000), whereby many systems have dominant males that court redd building females, however, variation occurs not only among species but

even between populations. Salmonid breeding systems vary in the degrees of mating competition, the degrees of sexual selection acting upon them and in the development of secondary sexual characters. This means that skews in the individual reproductive success exist in many salmonid breeding systems. Some systems have competition between large dominant males, usually with sumptuous secondary sexual characters, and small early matured male sneakers as the Atlantic salmon (Jones and King 1949) and coho salmon (Gross 1991), whereas other systems show a low degree of secondary sexual characteristics and less mating competition and sexual selection, as brown trout (Serbezov *et al.* 2010) and grayling (Fabricius and Gustafson 1955).

The species studied here is the European grayling, *Thymallus thymallus* (Linnaeus, 1758), hereafter referred to as grayling. Grayling is a spring spawning iteroparous salmonid freshwater fish. At present, there is little knowledge on the grayling breeding system, as most of the literature available on the subject are derived from purely observational studies that were performed at a time when it was not possible or practical to perform large scale genetic analyses on breeding populations (Fabricius and Gustafson 1955; Poncin 1994; Northcote 1995; Poncin 1996; Darchambeau and Poncin 1997). These observational studies propose that grayling has a polygynandrous mating system where both sexes mate with more than one partner. Opposite of most other salmonid species it is the males guard the spawning territories, and is approached by females. The male will defend their territory and attack other males and unripe females intruding.

The aims of this study were to investigate different aspects of the grayling breeding system in a small stream-spawning grayling population using a genetic approach trying to i) test if grayling indeed exhibit a polygynandrous mating system, as suggested by Fabricius and Gustafson (1955), and ii) to estimate individual reproductive success and potential causes for such individual differences in both sexes. Since male grayling are territorial during their spawning migration (Fabricius and Gustafson 1955; Poncin 1996), it is reasonable to assume that larger males may hold higher quality territories and therefore will be preferred by the females. The larger males would then be expected to mate with a large number of partners and produce a higher number of offspring than smaller individuals, resulting in a higher individual reproductive success. Regarding the female reproductive success, fecundity increases with female body size (Fleming 1998; Wootton 1998; Haugen 2000b), thus the larger females are expected to have higher individual reproductive success than smaller individuals.

The recent advances in the field of molecular genetics were utilized to investigate the grayling breeding system. It is now possible to use highly variable genetic markers, such as microsatellite loci, to perform assignments of parentage based on the genotype frequencies of progeny and potential parents. This can reveal details about the reproductive biology and the breeding systems of animals that are elusive in observational studies. This study utilized 19 microsatellite loci, from non-coding regions of the DNA (described in detail in Table 1; see also (Diggs and Ardren 2008; Junge *et al.* 2010), previously used to study the lake Lesjaskogsvatnet grayling "metapopulation" (Junge *et al.* 2011). Those loci were used to genotype the sampled grayling and the genotypes were in turn used for parentage assignments.

2 Material and methods

2.1 Study species

The European grayling (*Thymallus thymallus*; Figure 2) is a non-anadromous iteroparous freshwater fish belonging to the Salmonidae family (Northcote 1995) that is of both commercial and recreational importance. It is found in its natural habitat in most of northern Europe (Northcote 1995), from 42˚35'N to nearly 70˚N and from 65˚E to 5˚W (Mills 1971). Grayling is mainly found in cool and oxygen rich rivers, streams and lakes (Muus 1968; Mills 1971), but some populations even inhabit the brackish water of the northern Baltic Sea (Swatdipong *et al.* 2010). By appearance grayling is characterized by a small mouth, a pointed head and a high and large dorsal fin (Muus 1968; Maitland and Campbell 1992). Further general information on grayling can be found in the review by Northcote (1995). The grayling breeding system is, as mentioned earlier, still poorly understood. What is known is that unlike most other members of the Salmonidae family, grayling are spring spawners (Maitland and Campbell 1992). In Norway, grayling have been quite intensely studied and it has been shown that for example lake living mature grayling exhibit varying degrees of homing, and migrate either to their tributary of birth or a neighbouring one for spawning, as observed after the ice breaks, for example by Kristiansen and Doving (1996), at temperatures above 4-7˚C (Muus 1968; Northcote 1995). Grayling also differ from most other salmonid species regarding the fact that it is the male, and not the female, that finds and guards the territory used for spawning (Fabricius and Gustafson 1955; Garant *et al.* 2001). Grayling are highly aggressive during the spawning season and males are fighting quite frequently, attacking both trespassing males and unripe females. Thus, it is assumed that the larger and the more of a competitive fighter a male is, the better spawning ground it is able to obtain (Fabricius and Gustafson 1955). Good spawning sites for grayling are typically shallow pools with moderate water currents and, often pea-sized, gravel beds (Fabricius and Gustafson 1955; Maitland and Campbell 1992). Fabricius and Gustafson (1955) studied the spawning behaviour of grayling visually and described it in the following way; "When the female is quite ripe, she approaches the male, showing a posture of readiness, in which she arches her back and presses her dorsal fin down. The male responds by tilting over on his side, covering the back of the female by his big dorsal fin, bending his tail across the tail of the female and trembling, and the mating act follows. During the spawning act, the female bends the caudal

part of her body dorsoventrally in such a manner that her tail is lifted, and works her genital opening deep down into the gravel by vigorous vibrating movements. The eggs are released under the surface of the gravel. … Thus, the courting, the nest-digging, and the mating are combined in the grayling into one action, unlike salmon, trout and char, in which they are separate activities." In addition Fabricius and Gustafson (1955) observed that grayling had a promiscuous or polygynandrous mating system, where both sexes mated with more than one partner. During spawning the eggs are usually buried up to 4 centimetres beneath the gravel surface (Fabricius and Gustafson 1955), were they hatch up to 40 days after their fertilization. The alevins remains in the gravel for up to 10 days, when the yolk sac is resorbed (Bardonnet *et al.* 1991). After swim-up, when the larvae emerges from the gravel and becomes free-living, it might stay in its tributary for up to 1.5 month before the, by then called, fry migrates or drifts downstream to the lake. Female grayling can lay between 421 – 36,000 eggs per breeding season (Hendry and Stearns 2004).

Figure 2: Male adult grayling from Søre Skottåe being measured for fork length.

2.2 Study site

The lake Lesjaskogsvatnet (Figure 3) is a shallow (mean depth of 10 metres) mountain lake (611 meters above sea level) with a surface area of approximately 4.52 km^2 and is about 10 km long. The lake serves as the headwaters for two large river systems, namely Gudbrandsdalslågen to the east and Rauma to the west. The lakebed mainly consists of pre-

eucambrian granitic gneiss which gives low-conductivity water (8 – 18 µS cm^{-1}), with a pH between 6.2 – 7 and high Secchi-depths ranging from 7 to 10 meters (Haugen 2000a). Grayling were introduced to the Lake Lesjaskogsvatnet system at the end of the 19th century by humans (Haugen and Vøllestad 2001), and has been subject to a number of different studies (Haugen and Vøllestad 2000; Haugen and Vøllestad 2001; Gregersen 2005; Gregersen *et al.* 2008; Barson *et al.* 2009; Junge *et al.* 2011; Thomassen *et al.* 2011). In addition to grayling, both brown trout, *Salmo trutta*, and European minnow, *Phoxinus phoxinus*, are found in Lesjaskogsvatnet (Haugen 2000a).

Søre Skottåe (highlighted in Figure 3) is one of 28 spawning tributaries used by grayling in Lesjaskogsvatnet. It is a small stream, 1 – 1.5 meters wide, found in the northeast end of the lake and it has a mean June temperature of 6˚C (Gregersen *et al.* 2008). The stream has some stretches were the water is flowing at a medium speed over bottom material consisting of fine gravel and cobble, and some stretches were the water is slow-flowing over a sand bottom (Gregersen 2005). Each year several hundred grayling may ascend the stream for spawning during May-June (Gregersen *et al.* 2008).

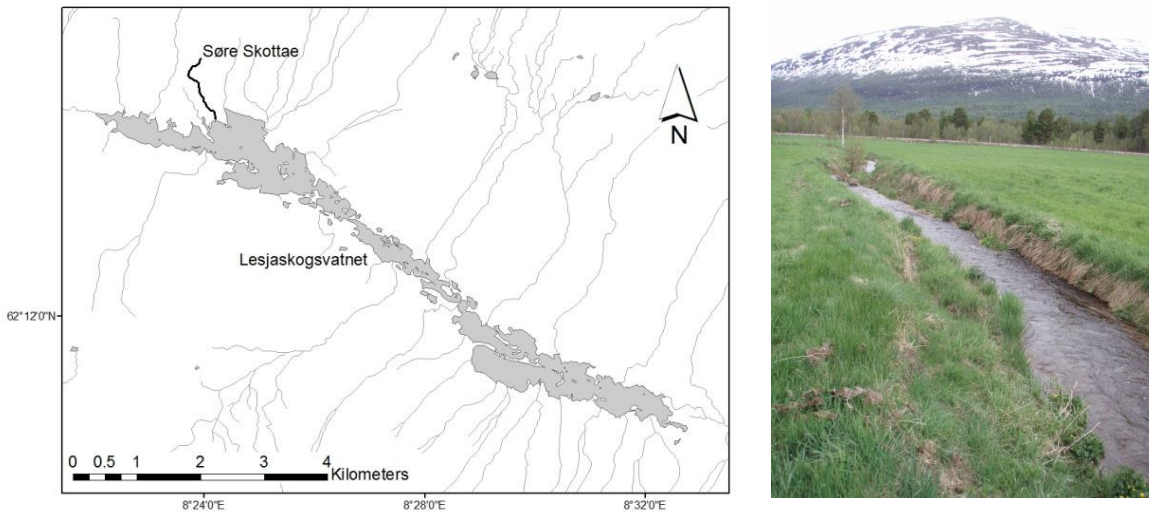

Figure 3: Map of Lesjaskogsvatnet with the stream Søre Skottåe highlighted (left), and a photography of Søre Skottåe during early summer showing how it runs through an agricultural landscape (right).

2.3 Sampling and genotyping

2.3.1 Sampling

Grayling were sampled in Søre Skottåe, between May and July in 2008. Mature grayling were caught with fyke nets (Figure 4, right) during their upstream spawning migration. All fish were anesthetized with clove oil (Mylonas *et al.* 2005), had their fork length measured (nearest mm), and they were sexed based on external sexual characters. Further, fin clips were excised from the adipose fin and then stored in numbered 2.0 mL eppendorf tubes filled with 96% ethanol for later genetic analysis. The individuals were allowed to recover from the anaesthesia before they were released back to the stream upstream for the nets to complete their spawning.

Grayling fry were sampled with drift nets (Figure 4, left) in the beginning of July 2008. A number of drift nets were deployed to cover most of the stream to collect larvae drifting downstream with the current. No quantification of drift was performed. It is assumed that the sampled fry is a random sample of all downstream drifting fry. The fry were first stored in batches and then separated individually into numbered 2.0 mL eppendorf tubes with 96% ethanol for preservation until later DNA isolation. In total 895 individual grayling fry and 149 mature grayling, 54 females and 95 males, were sampled.

Figure 4: Left; sampling grayling fry that is drifting out of the stream with drift nets facing upstream. Right; sampling adult grayling on their spawning migration using a fyke net facing downstream.

2.3.2 DNA isolation

DNA from most of the sampled grayling, adult and fry, were extracted using a method after Aljanabi and Martinez (1997), and the procedure is described below. The lengths of the fry were measured to the nearest 0.5 millimetre using millimetre paper and a stereo microscope. The fry were then divided into two halves with sterile scalpels and pincers, were one half was used for further genetic analysis and the other as a backup source of DNA. The tissue pieces were placed in labelled 1.5 mL eppendorf tubes containing 200 µl salt extraction buffer, consisting of 0.4 M NaCl, 10 mM Tris-HCl with a pH of 8.8 and 2 mM Ethylenediaminetetraacetic acid (EDTA) with a pH of 8.0, and 20 µl of 20% sodium dodecyl sulphate (SDS). Then 8µl of 20 mg/ml proteinase K digestive enzyme was added to the tubes before they were quickly vortexed. The tubes were then left in a shaker holding 60 ˚C for between 4 and 12 hours depending on how quick the samples dissolved, and the samples were taken out and vortexed a couple of times to aid tissue lysis. When the samples had dissolved, 150 µl of 6 M NaCl were added and gently mixed in by inverting the tubes. As soon as the NaCl was properly mixed, the samples were spun at 10600 rpm in a centrifuge for 32 minutes before 300 µl of the supernatant were removed and placed in labelled sterile 1.5 ml eppendorf tubes. 300 µl isopropanol were then added into each tube before they were placed into the freezer at -20˚C for at least one hour. After this treatment, the samples were centrifuged at 4˚C at a speed of 13000 rpm for 20 minutes followed by pouring out the isopropanol so that only the DNA pellets were left in the tubes. Next, 200 µl of ice-cold 70% ethanol was added before they were spun down at 4˚C for 8 minutes at 13000 rpm. The ethanol was then poured out of the tubes and then any remaining ethanol was allowed to evaporate by leaving the tubes open on a 60˚C heat block for 20 minutes or overnight at room temperature. The dried pellets were then eluted in 70µl of sterile H_2O by gently mixing the sample with a pipette (Aljanabi and Martinez 1997). Negative controls were used throughout the extraction process. For the remaining fish, the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used to extract the DNA according to manufacturer's protocol. All of the extracted DNA samples were stored in a -20˚C freezer until they were used in polymerase chain reactions and then genotyped.

2.3.3 Polymerase Chain Reactions (PCR)

The polymerase chain reactions or PCR amplifications of the 19 polymorphic microsatellite loci were performed in seven different reactions, two single and five multiplex PCRs (for details see Table 1 and (Junge *et al.* 2011)), to maximize multiplexing and therefore minimize the amount of time and running costs. One of four different fluorescent dyes (NED, VIC, PET or FAM) was used at each locus (Table 1). In short, the PCRs had annealing temperatures ranging from 58˚C to 60˚C and each individual reaction consisted of 4.25 µl of Qiagen multiplex PCR master mix, 1.5 µl of DNA, a specific concentration of the respective forward and reverse primer and sterile H_2O to reach a total volume of 7 µl in each well. The three different thermal cycling programs for the seven PCRs were identical except for the annealing temperatures, and were: 95˚C for 15 minutes, 94˚C for 30 seconds, 58- 60˚C for 1 minute and 30 seconds, 72˚C for 1 minute, followed by 37 cycles of 94˚C for 30 seconds, then 60˚C for 30 minutes, followed by 20˚C for 5 minutes.

Table 1: Details of the microsatellite loci and primers used in the study. The locus information is the common name and the relevant publication or GenBank accession number if unpublished. Label is the fluorescent dye used. MP states which multiplex group the locus was amplified in or if it was single locus PCR amplification. Primer is the primer concentration in the PCR-mix. PCR states the annealing temperature of the PCR. Na is the number of alleles found in the locus. Allele-range is the range of the length of alleles in base pairs.

Locus information			**Amplification details**			**Genetic diversity**	
Reference	*Locus*	*Label*	*MP*	*Primer*	*PCR*	*Na*	*Allele-range*
GenBank: AF151370	BFRO13	FAM	MP1	0.09	58	4	235-247
(Junge *et al.*, (2010))	213	FAM	single	0.38	60	11	283-327
(Junge *et al.*, (2010))	414	FAM	MP2	0.20	60	6	393-413
(Junge *et al.*, (2010))	309	FAM	MP4	0.57	59	2	447-451
(Diggs *et al.*, (2008))	TAR106	FAM	MP5	0.07	59	8	193-221
(Sušnik *et al.*, (2000))	BFRO10	VIC	MP1	0.08	58	2	96-122
(Sušnik *et al.*, (1999b))	BFRO15	VIC	MP1	0.04	58	2	144-154
(Sušnik *et al.*, (1999b))	BFRO18	VIC	MP1	0.04	58	4	181-195
(Junge *et al.*, (2010))	207	VIC	MP1	0.10	58	2	216-224
(Sušnik *et al.*, (1999a))	BFRO9	VIC	MP1	0.05	58	2	243-247
(Junge *et al.*, (2010))	438	VIC	single	0.34	60	9	265-297
(Sušnik *et al.*, (2000))	BFRO11	NED	MP3	0.30	59	2	86-102
(Junge *et al.*, (2010))	313	NED	MP2	0.18	60	6	180-200
(Olsen *et al.*, (1998))	Ogo2	NED	MP1	0.07	58	3	233-241
(Junge *et al.*, (2010))	433b	NED	MP3	0.18	59	8	287-315
(Junge *et al.*, (2010))	445	NED	MP4	0.13	59	12	374-422
(Junge *et al.*, (2010))	415	PET	MP3	0.33	59	9	193-225
(Junge *et al.*, (2010))	214	PET	MP1	0.14	58	4	292-313
(Junge *et al.*, (2010))	407b	PET	MP5	0.20	59	7	230-254

2.3.4 Electrophoresis and scoring

All PCR products from one individual were combined and subsequently diluted 1:40. 2 µl of those combined and diluted PCR products were added to a 10:1 mix of formamide and GeneScan™ - 600 LIZ® size standard (Applied Biosystems, Foster City, CA), reaching a total volume of 12 µl in each well. This was then electrophoresed on a 48-capillary ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA). After the electrophoresis, the data was analysed and the genotypes were scored using GeneMapper® 4.0 software (Applied Biosystems, Foster City, CA). Positive controls were included on a regular basis and all the scored alleles were visually checked after the automated scoring process to minimize the amount of scoring errors and maximize accuracy. A total of 840 individual grayling fry and 149 individual mature grayling were successfully genotyped for at least 16 of the 19 microsatellite loci (1 at 16 loci, 5 at 17 loci, 1 at 18 loci and 983 at all 19 loci).

2.4 Analyses

2.4.1 Loci and grayling characteristics

GenAlEx 6.41 (Peakall and Smouse 2006) was used to perform a multilocus match analysis for codominant data to ensure that no individual grayling appeared more than once in the data set. This was particularly important in this study as some of the fry were retrieved only as pieces from the drift nets. CERVUS 3.0 (Marshall *et al.* 1998; Kalinowski *et al.* 2007) was used to calculate the polymorphic information content (PIC) of each of the 19 microsatellite loci The PIC value of a locus is calculated from the allele frequencies, and the PIC is a measure of the information content and variation at each locus. GenAlEx was further used to calculate the unbiased expected heterozygosity and the observed heterozygosity and to check for potential deviations from Hardy-Weinberg equilibrium (HWE).

2.4.2 Parentage assignment

The parentage assignments were performed by two computer programs, COLONY 2.0 (Wang 2004; Wang and Santure 2009; Jones and Wang 2010) and CERVUS 3.0 (Marshall *et al.* 1998; Kalinowski *et al.* 2007), using the genetic data from the genotyped adult and fry grayling. I decided to use two different methods of parentage analysis to strengthen the

assignments and to be able to compare them (Karaket and Poompuang 2012). The two computer programs differ in their assumptions and in how they make the assignments. CERVUS uses a pairwise likelihood comparison method when assigning offspring to parent pairs. It produces locus-by-locus likelihood scores for each potential parent for each offspring and then assigns parentage to the candidate parents with the highest combined score over all genotyped loci. It also gives the confidence of each parentage assignment as relaxed (80%) or strict (95%), only the assignments made under a strict confidence were used in this study. COLONY has a different approach when assigning parentage as it simultaneously infers both sibship and parentage among individuals with full-pedigree likelihood methods using the multilocus genotype data. Contrary to CERVUS, COLONY considers the likelihood of the entire pedigree configuration and not by parent pair.

I performed several parentage analyses with both programs using different settings to optimize the running conditions and to check for errors and minimize them. All the assignments for both the programs were run with 840 offspring, 54 female and 95 male grayling genotyped at 19 loci, where the adults where imported as potential fathers or mothers. The parameters used in the "best model" parentage assignment from CERVUS that were used in the study were as follows; number of simulated offspring = 5000, proportion of parents sampled = 90%, proportion of loci genotyped = 99.9%, proportion of genotype errors (mistyped) = 1%, the error rate in likelihood calculations = 1% and the confidences were determined using DELTA scores. The parameters for the "best model" parentage assignment with COLONY were as follows; proportion of parents sampled = 90%, allelic dropout rate = 0.5%, rate of other kinds of genotyping errors (including mutations) = 0.5%, mating system = polygamy (for both sexes), species = diploid, seed for random number generator = 1234, length of run = medium, and likelihood precision = very high.

The assignments used to investigate the grayling breeding system were run with the "best model" settings, which were chosen as they resulted in the most optimal results and represented the settings that reflected the sampling in the best way. It should be noted however, that there were no fundamental differences in the results produced with the different inputs and settings.

2.4.3 Statistical analyses

Negative binomial generalized linear model using MASS (Venables and Ripley 2002) in R (R development core team 2012) were used to test the relationships between the individual reproductive success, and fork length and timing of spawning migration. This model was chosen as it was a better fit to the data from the parentage assignments than a Poisson regression.

2.5 Ethics

Animal sampling and experimentation were performed in compliance with the recommendations of National Animal Research Authority (permission ID 2008/7368.5) and under the supervision of authorized investigators

3 Results

3.1 Loci and grayling characteristics

3.1.1 Grayling characteristics

Adults: 95 male and 54 female adult grayling were sampled during the spawning migration in the Søre Skottåe tributary over a 10-day period in late May and early June 2008 (Figure 5). Male grayling were significantly larger than female grayling (Welch two sample t-test, t = -8.62, p-value < 0.001; see Figure 6), with males ranging from 248 to 416 mm (mean ± SD, 320.9 ± 28.0 mm) in fork length and females ranging from 250 to 330 mm (287.8 ± 18.7 mm).

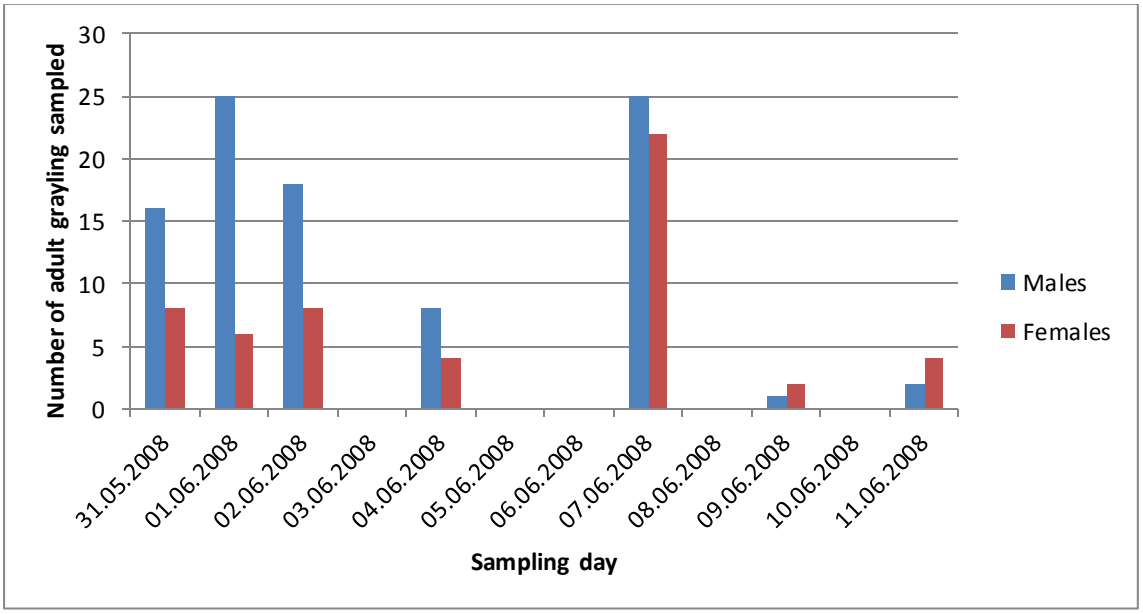

Figure 5: Number of adult male and female grayling sampled at each sampling day (month/day/year).

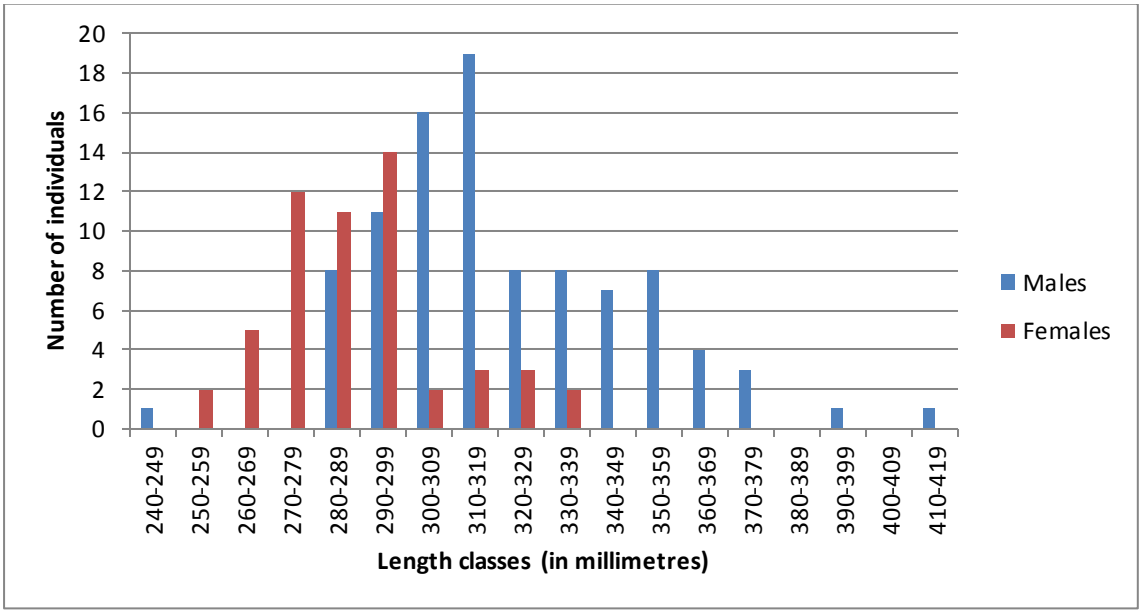

Figure 6: The adult grayling fork length distribution in Søre Skottåe sampled during 2008.

Fry: Of the 895 sampled and genotyped fry, the multilocus match analysis for codominant data in GenAlEx revealed that 45 genotypes had to be removed as their exact genotype already appeared in the data set (Probability of identity over 19 loci = 7.5E-14). Consequently, 840 unique fry genotypes were analysed in this study. A total 530 fry were measured for length to the nearest 0.5 mm. Mean fry length was 13.9 ± 1.5 mm, ranging from 8 to 17 mm (Figure 7). Fry were sampled over a period of approximately three weeks, with the majority being caught over a two-day period on July 9-10.

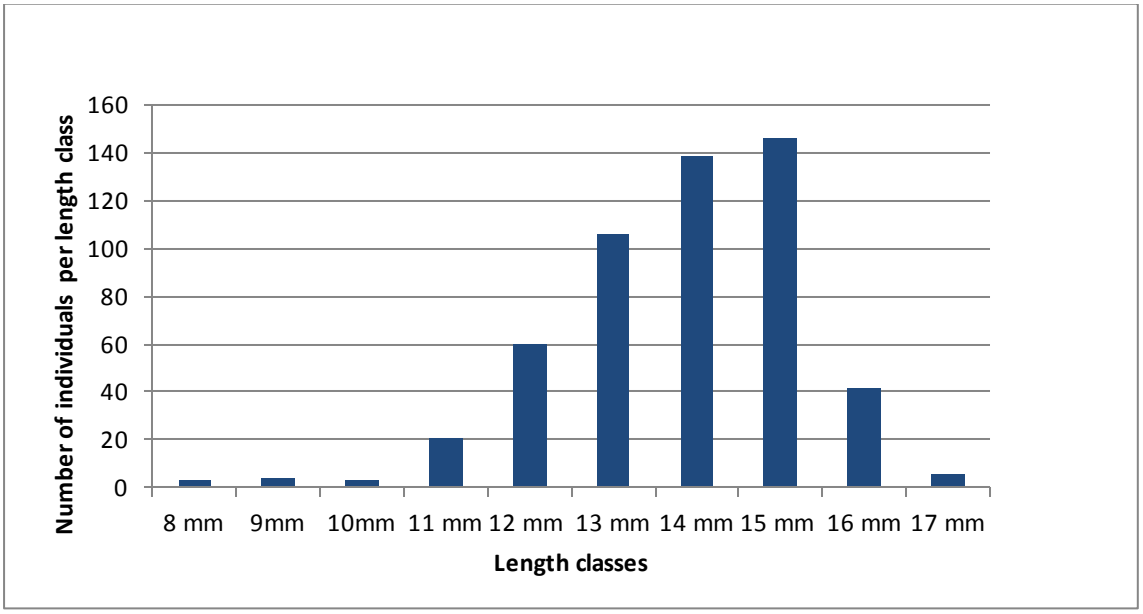

Figure 7: Length distribution of grayling fry sampled with drift nets in Søre Skottåe during July 2008.

3.1.2 Loci characteristics

The 19 microsatellite loci used in this study were investigated mainly in order to ensure that they revealed enough variance to be informative for the assignments, and further, that the assumptions made by the parentage assignments were not violated. The mean PIC (polymorphic information content) over all loci ranged from 0.15 to 0.82 (mean ± SD, 0.53 ± 0.19) in adults and from 0.21 to 0.82 (mean ± SD, 0.53 ± 0.18) in fry (see Table 2), and did not differ significantly between them (Figure 6; Welch two sample t-test, t = 0.0088, p-value = 0.99). The 19 loci were moderately to highly informative (2 loci below 0.3, 9 loci 0.3 – 0.59, 8 loci above 0.6). A PIC ranging from 0 – 0.29 is uninformative, a PIC between 0.3 – 0.59 is considered moderately informative and a PIC above 0.6 is considered highly informative (Mateescu *et al.* 2005). The mean unbiased expected heterozygosity was 0.59 for both, adults and fry, and the mean observed heterozygosity was 0.60, also for both (see Table 2), with no significant differences detected between adults and fry (Figure 6; UHE: Welch two sample t-test, t = 0.018, p-value = 0.99) and the observed heterozygosity (Table 2; Welch two sample t-test, t = -0.036, p-value = 0.97) were investigated.

The allele frequencies of the sampled fry showed deviations from Hardy-Weinberg equilibrium ($P < 0.05$) in 11 of the 19 loci (BFRO13, Tth-213, TAR106, BFRO10, Tth-438, BFRO11, Tth-313, Ogo2, Tth-445, Tth-415 and Tth-407b) which is still significant after Bernoulli (Moran 2003), whereas the adult genotypes only deviated at one locus (Tth-407b)

which is not significant after Bernoulli (Moran 2003); Table 2). The 11 loci deviating in the fry genotypes were still included in the study as this was an expected result of sampling large family groups and the fry genotypes being results of only a "random" selection (only the successful adults) of the mature genotypes.

Table 2: Information on the 19 loci used, divided in fry and adults. Locus gives the common name of the locus. UH_E is the unbiased expected heterozygosity. H_O is the observed heterozygosity. H-W states the significance test for Hardy-Weinberg deviations; deviating loci are in bold. PIC is the polymorphic information content of each locus. The last row contains the average and the standard deviation of the heterozygosity and PIC across all loci.

Locus name	**Adult**				**Fry**			
Locus	*UH_E*	*H_O*	*H-W*	*PIC*	*UH_E*	*H_O*	*H-W*	*PIC*
BFRO13	0.67	0.71	0.21	0.59	0.67	0.70	**0.00**	0.62
Tth-213	0.79	0.83	0.27	0.76	0.78	0.80	**0.01**	0.75
Tth-414	0.72	0.72	0.98	0.68	0.66	0.62	0.29	0.61
Tth-309	0.45	0.40	0.20	0.35	0.44	0.46	0.12	0.34
TAR106	0.67	0.63	0.62	0.62	0.73	0.75	**0.00**	0.68
BFRO10	0.35	0.36	0.72	0.29	0.32	0.36	**0.00**	0.27
BFRO15	0.50	0.52	0.68	0.38	0.49	0.49	0.95	0.37
BFRO18	0.56	0.59	0.70	0.47	0.56	0.60	0.18	0.47
Tth-207	0.48	0.52	0.31	0.36	0.50	0.51	0.38	0.37
BFRO9	0.17	0.17	0.82	0.15	0.24	0.23	0.27	0.21
Tth-438	0.80	0.83	0.90	0.77	0.81	0.77	**0.00**	0.78
BFRO11	0.45	0.46	0.68	0.35	0.46	0.51	**0.01**	0.35
Tth-313	0.74	0.77	0.89	0.69	0.71	0.71	**0.00**	0.67
Ogo2	0.64	0.67	0.78	0.56	0.64	0.64	**0.00**	0.56
Tth-433b	0.66	0.60	0.33	0.61	0.58	0.60	0.11	0.53
Tth-445	0.84	0.86	0.93	0.82	0.84	0.85	**0.00**	0.82
Tth-415	0.76	0.76	0.79	0.72	0.76	0.76	**0.00**	0.72
Tth-214	0.50	0.51	0.27	0.45	0.49	0.50	0.82	0.44
Tth-407b	0.44	0.39	**0.00**	0.42	0.49	0.46	**0.00**	0.46
Mean	*0.59±0.18*	*0.60±0.19*	*n/a*	*0.53±0.19*	*0.59±0.17*	*0.60±0.16*	*n/a*	*0.53±0.18*

3.2 Parentage assignment

The genotyped grayling, 840 fry and 149 adults, which were sampled during the spring and summer of 2008, were used to gain investigate a small stream grayling breeding system.

I used two computer programs, COLONY and CERVUS, to assign parentage to the adult grayling with the earlier described "best model" parameters. *Parent pair assignment*: CERVUS assigned 210 offspring (25%) with both a father and a mother at a probability above 95% whereas COLONY assigned a parent pair to all 840 offspring (100%; Table 3). *One parent assignment*: When either assigning offsprings to a sampled father, or mother, at a

probability above 95%, CERVUS assigned 480 offspring (57%) with a father and 329 offspring (39%) with a mother, whereby COLONY assigned 565 offspring (67%) to a father and 195 offspring (23%) to a mother (Table 3).

Table 3: Summary of the number of adult male and female grayling with reproductive success estimated with the two parental assignment models (COLONY, CERVUS). Parent pair means that both a father and a mother were assigned to an offspring. One parent means that either a father or a mother was assigned to an offspring.

	Male		**Female**	
	Parent pair	*One parent*	*Parent pair*	*One parent*
COLONY	33.7% (32 of 95)	33.7% (32 of 95)	33.3% (18 of 54)	31.5% (17 of 54)
CERVUS	45.3% (43 of 95)	50.5% (48 of 95)	81.5% (44 of 54)	85.2% (46 of 54)

3.3 Statistical analyses

3.3.1 Mating success

To investigate how many mating partners a successfully breeding grayling had, I investigated the results where an offspring was assigned with a parent pair, and not only a father or a mother, in more detail. The results from the two programs differed, as expected, in the number of partners for males and females but both the results from COLONY and from CERVUS showed that both male and female grayling on average mated with more than one partner in the 2008 spawning season (Figure 8). Successfully reproducing males (33.7%) had on average of 17.7 ± 18.2 (standard deviation (SD)) offspring with an average of 4.1 ± 3.4 (SD) partners (ranging from 1 – 74 offspring and 1 – 13 partners). The successful females (33.3%) had on average 10.9 ± 13.1 (SD) offspring with an average of 2.3 ± 1.7 (SD) partners (1 – 46 offspring and 1 – 6 partners).

CERVUS only assigns parentage to sampled adults and does not infer parents. The obtained results, however, also showed an average of more than one mate for both sexes (Figure 8). Successfully reproducing males (45.3%) had on average 4.9 ± 6.0 (SD) offspring with on average 2.9 ± 2.5 (SD) partners (ranging from 1 – 28 offspring and 1 – 10 partners).

The successful females (81.5%) had on average 4.8 ± 5.0 (SD) offspring with on average 2.8 ± 2.3 (SD) partners (1 – 19 offspring and 1 – 10 partners).

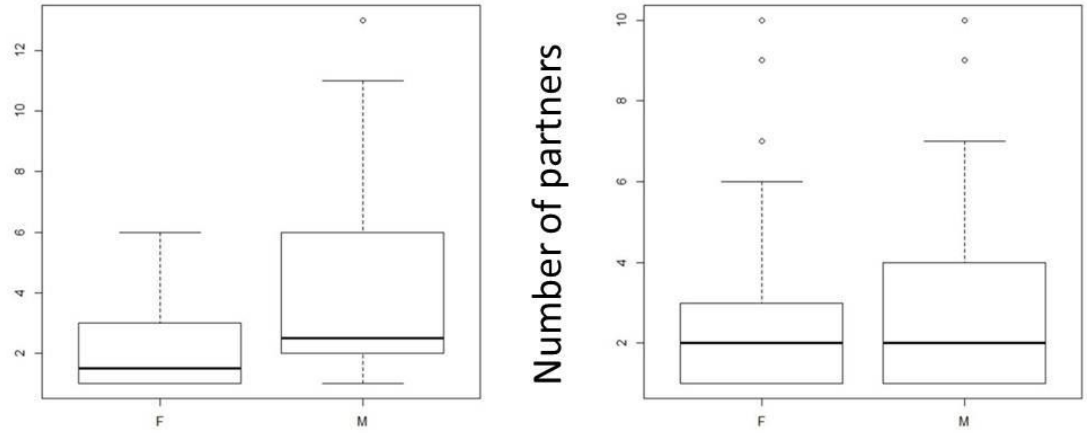

Figure 8: Box and whisker plots showing the number of partners of the reproductively successful adult grayling (F: female, M: male). Bold line representing the median and dots representing outliers. Results from: COLONY (Females: n = 18, males n = 32; left) and CERVUS (Females: n = 44, males n = 43; right)

3.3.2 Reproductive success

The results from both parentage assignments showed large variation in individual reproductive success for both males and females (Table 3 and Figure 8). To test if (1) the size of the adult breeders or (2) the timing of an individual's spawning run could explain the observed variation, I used the results from the parentage assignments where either a mother or a father was assigned, excluding the unsuccessful individuals (63 of 95 males and 37 of 54 females from COLONY and 47 of 95 males and 8 of 54 females from CERVUS).

3.3.3.1 Reproductive success vs. fork length

Overall, there was large individual variation in reproductive success for either sex using either of the two programs, and there was no strong relationship between fish size (i.e. fork length) and success (COLONY:

Figure 9; CERVUS: Figure 10). For the females, there was no clear indication of the size – success relationship for either assignment method (GLM, negative binomial regression; COLONY: effect ± SE = -0.021 ± 0.014, DF = 16, p-value = 0.14, CERVUS: effect ± SE = -0.0051 ± 0.0076, DF = 45, p-value = 0.51). However, there was a tendency for the larger

males to have more offspring, but the size-success relationship was only significant for the CERVUS assignments (GLM, Negative binomial regression; COLONY: effect ± SE = 0.0053 ± 0.0050, DF = 31, p-value = 0.29, CERVUS: effect ± SE = 0.015 ± 0.0049, DF = 47, p-value = **0.0017**).

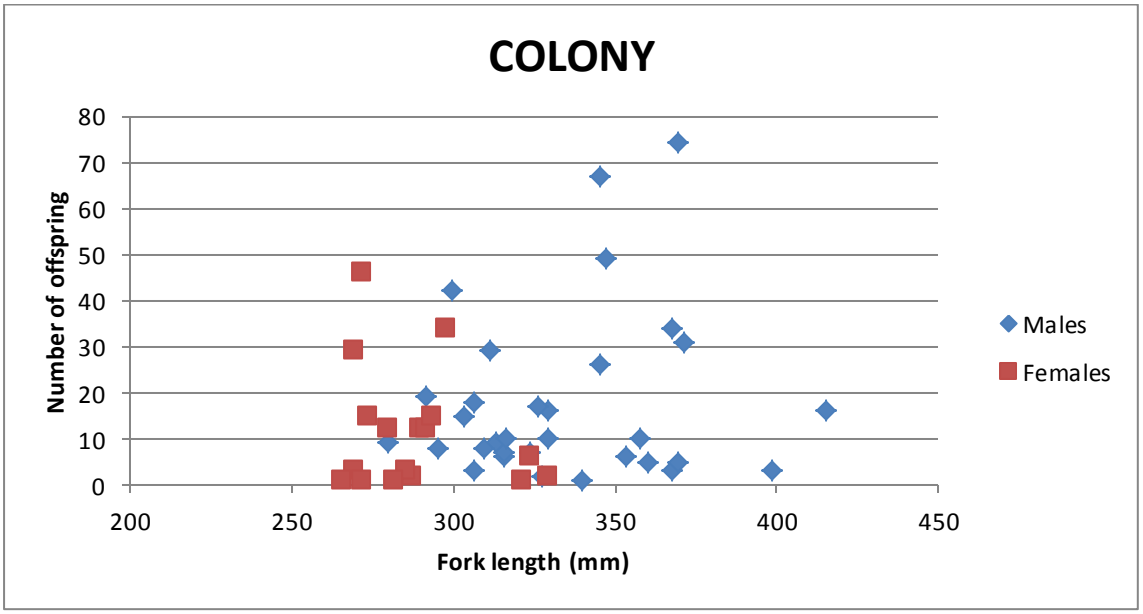

Figure 9: Number of offspring in relationship to own body size (stated as fork length) based on assignment results from COLONY.

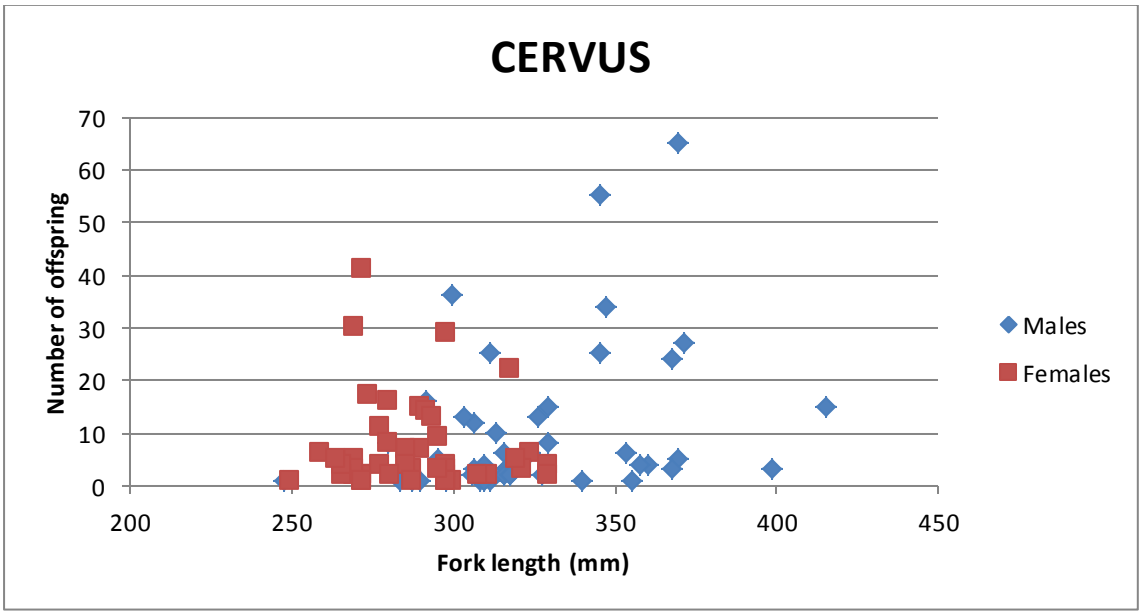

Figure 10: Number of offspring in relationship to own body size (stated as fork length) based on assignment results from CERVUS.

3.3.3.2 Reproductive success vs. timing of spawning migration

There was no relationship detected between timing of the spawning migration and the individual reproductive success with either of the two programs. This was furthermore irrespective of the sex (GLM, Negative binomial regression; males: COLONY: effect ± SE = -0.027 ± 0.055, DF = 31, p-value = 0.63 and CERVUS: effect ± SE = -0.071 ± 0.056, DF = 47, p-value = 0.21; females: COLONY: effect ± SE = 0.019 ± 0.090, DF = 16, p-value = 0.83 and CERVUS: effect ± SE = 0.0082 ± 0.044, DF = 45, p-value = 0.85) (Figure 11 and Figure 12).

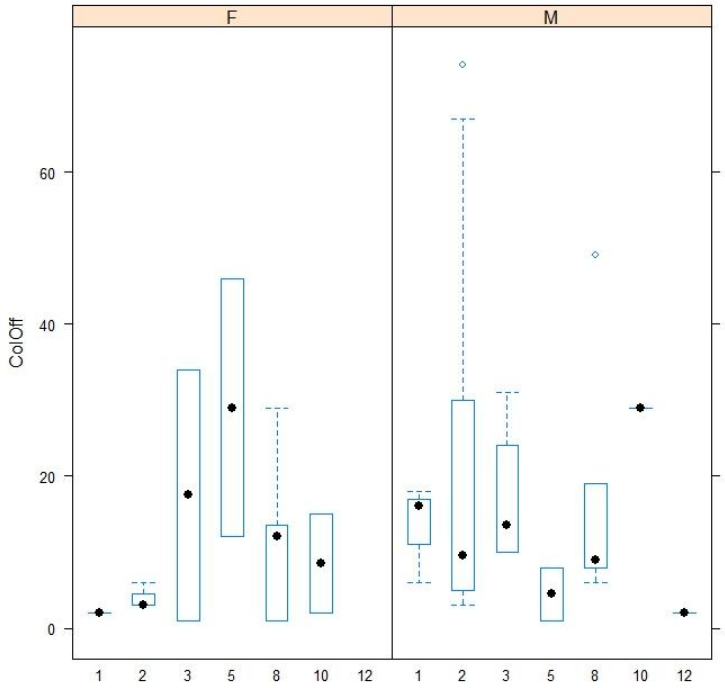

Figure 11: Boxplot showing number of offspring (y-axis) vs. the timing of spawning migration (i.e. day sampled; x-axis) for females (F) and males (M) with the results from COLONY.

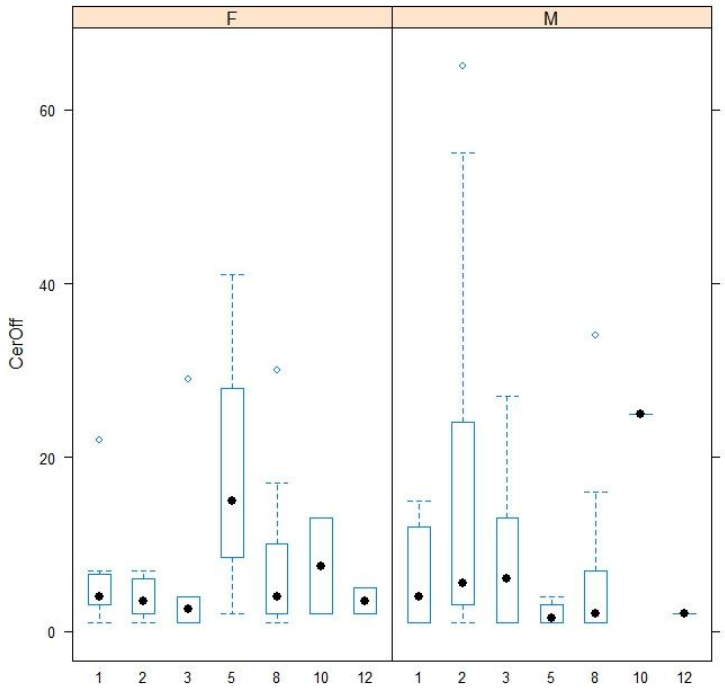

Figure 12: Boxplot showing number of offspring (y-axis) vs. the timing of spawning migration (i.e. day sampled; x-axis) for females (F) and males (M) with the results from CERVUS.

4 Discussion

The genetic investigation of the European grayling breeding system revealed that grayling exhibit a polygynandrous mating system within a small tributary. This has previously been suggested through behavioural studies (Fabricius and Gustafson 1955; Poncin 1994; Poncin 1996; Darchambeau and Poncin 1997), but has never been shown with genetic tools, which allow the follow-up of the actual mating outcome. Both males and females were successfully producing offspring with more than one partner, and their individual reproductive success was skewed. This observed individual variation, could neither be explained by body-size nor timing of the spawning run. Although the males with the highest number of offspring were longer than average, it was only for the fathers assigned by CERVUS that a significant positive relationship between body length and the number of offspring produced, i.e. reproductive success, could be detected. COLONY and CERVUS produced different parentage results, which may be explained by their difference in the approach to assign parentage.

Polygynandry

The detection of a polygynandrous mating system in grayling through this genetic investigation is in concordance with previously conducted observational studies. This study shows the first evidence of male and female grayling not just mating, but also successfully producing offspring with more than one partner. The polygynandrous mating in grayling were first described by Fabricius and Gustafson (1955) who observed grayling of both sexes mating with more than one partner during their spawning run, but it has so far never been now been genetically confirmed.

As it is energetically less expensive to produce sperm than eggs (Hendry and Stearns 2004) it is common that males have more partners than females, as females have to be choosier in which males they invest. The advantages of reproducing with more than one partner may be; to increase the genetic diversity, to spread the redds at different locations (male territories) as insurance against outer factors or simply to spread the genes (Neff and Pitcher 2005). It could also be an effect of female's limited ability to evaluate their partners quality, and thus try to enhance their reproductive success by mating with several males

(Yasui 1998). One important disadvantage with a multiple partner mating strategy is that an individual may by chance only mate with lower quality partners.

Parentage assignments

The parentage assignments conducted in the two programs COLONY and CERVUS were the basis for all further estimations and therefore crucial for this study of the grayling breeding system. Therefore, results of both programs were carefully evaluated and running parameters changed and adapted until the performance was judged as optimal, given the genotype data present. Overall, both programs used for parentage assignment in this study revealed the same major findings and trends. Individual differences in assignment results between COLONY and CERVUS are most likely a consequence of the fact that the programs are using different approaches when assigning parentage. CERVUS uses a pairwise likelihood comparison method to assign parentage to the possible parents, while COLONY assigns parentage with full-pedigree likelihood methods. COLONY further infers parent genotypes (in this study, 17 fathers and 41 mothers) where a matching parent genotype is absent from the data set of sampled possible parents. This could have consequences for the estimation of the percentage of reproductively successful individuals as well as the number of mating partners. The inference of additional parental genotypes leads to an estimated fewer percentage of sampled individuals apparently contributing with offspring due to a higher number of adults overall. This could therefore be the reason for the differences in the results with respect to the number of reproductively successful individuals (Percentage with individual reproductive success: COLONY; males = 33.7%, females = 31.5%, CERVUS; males = 50.5%, females = 85.2%). It can also lead to higher estimated numbers of mating partners, as sampled individuals are also paired with inferred partners. This method-dependent bias would be particularly strong for the males, simply because more males were sampled that could have mated and will be used to construct more female genotypes (Average number of mating partners: COLONY; males = 4.1, females = 2.3, CERVUS; males = 2.9, females = 2.8; Figure 8). However, with the exception of male length explaining variance in reproductive success in the results from CERVUS, the two programs results lead to the same conclusions. Both programs recognize the same individuals as the ones with the highest number of offspring (although the number differs slightly).

Possible factors that could have led to general biases or imprecise estimations could include insufficient sampling of adults and/or fry. In total, 149 adults were sampled. If this represented an insufficient proportion of the breeding population it could have strongly affected the precision of the estimates (see (Serbezov *et al.* 2010). In a previous study comprising this spawning population, the effective population size has been estimated to be 63 (CI: 40–126; (Junge *et al.* 2011)). Although the actual number of breeders is assumed to be higher than the number of effective breeders, it is still unlikely that the Søre Skottåe grayling represent a very large population, and therefore that the sampling was very insufficient representing only a very small and random sample of the actual breeding population. Further, for this study, samples for only one breeding season were available to be analysed, which has been shown to result in many drawbacks (Serbezov *et al.* 2010). Another source of difference between the results is that the sexing of the fish was done by eye and therefore errors may have occurred. If grayling were wrongly sexed it would affect COLONY's results more, as the wrongly sexed individuals would lead to more inferred parents and artificial lower number of reproducing individuals.

Reproductive success

There was a large variation in individual reproductive success in both male and female grayling. This reproductive skew means that some individuals, for different reasons, produce a lot more offspring than others do. It was expected to be a larger reproductive skew for males as this is observed in other salmonids (Hendry and Stearns 2004), but this gender difference was not found in this study. In the results from CERVUS, reproductively successful males had 4.9 offspring with 2.9 partners. However, it appeared to be a larger reproductive skew in males in the results from COLONY, were reproductive successful males had 17.7 offspring with 4.1 partners and females had 10.9 offspring with 2.3 partners. But the result from COLONY is most likely not as reliable as the one from CERVUS since it was based on 32 males and only 18 females. Both the males and the females of salmonid species tend to show this variability, e.g. brown trout (Serbezov *et al.* 2010), brook trout (Blanchfield *et al.* 2003), pink salmon (Dickerson *et al.* 2004) and Atlantic salmon (Garant *et al.* 2001). The promiscuous mating system in grayling that is found in this study will decrease the amount of sexual selection as it leads to a high number of individuals with reproductive success compared to e.g. a polygynous mating system (Shuster 2009). However, in general, the variability in reproductive success is thought to be a response of tough mating competition

among salmonids during spawning, and this variation gives sexual selection something to act upon (Hendry and Stearns 2004). This means that despite the fact that grayling mate polygynandrously; the large observed intrasexual reproductive skew within both sexes might mean that there is sexual selection acting upon undetectable traits in both males and females. This study did not reveal large variance in the intersexual (that is between the sexes) reproductive skew, however, in other mating systems it is common that the reproductive skew is larger and thus sexual selection is higher in males (Andersson and Iwasa 1996).

The here detected variance in individual reproductive success could neither be conclusively explained with body length, even though at least the males that produced the highest number of offspring were above average in body length, nor with timing of the spawning run. However, there was a positive relationship between male body length and success in the results from the parentage assignments performed by CERVUS, but not in the results from COLONY. It was expected that larger grayling would have greater reproductive success compared to smaller ones, as large salmonids are more competitive fighters, more able to dig deep high quality redds and show signs of being of high quality because they have been able to grow large, which has been supported by observational studies (Fleming *et al.* 1996; Hendry and Stearns 2004). However, genetic studies on salmonid breeding systems often result in finding weak and noisy relationships between individual reproductive success and body length, e.g. brown trout (Serbezov *et al.* 2010), brook trout (Blanchfield *et al.* 2003) and Atlantic salmon (Garant *et al.* 2001). This does not mean that there is no reproductive gain for larger individuals, but merely that it is not straightforward to detect such a relationship. The fact that the stated increase in reproductive success by body length from observations and theory is hard to verify with genetic studies in salmonids is often due to a limited sample size (Garant *et al.* 2001). Reasons to why this relationship was not found in this study may comprise that polygynandrous species tend to have a minimal improvement in reproductive success as a response to increased body size (Avise *et al.* 2002), a strong preference for non measureable traits (Dickerson *et al.* 2004), or that the sample size were too low. The 149 adults sampled are acceptable in this small population, but the 840 fry sampled represent only a fraction of the fry that were produced in the stream in 2008. Taking it to the extremes, since female grayling are assumed to spawn between 421 to 36,000 eggs (Hendry and Stearns 2004), the 54 sampled mature females should have been able to produce anything between 23,000 to 1.9 million eggs. This means that the sampled 840 fry would represent only 0.04 % of the offspring in the worst and 3.7% in the best-case scenario, given that each

egg is fertilized and there is no offspring mortality. This is obviously a hypothetical calculation, but it illustrates that the proportion of fry sampled represents only a very small amount of the total offspring produced in 2008 in Søre Skottåe. Another issue with the samples in this study is that the grayling native to Søre Skottåe and the neighbouring stream Steinbekken migrate between them during mating (Junge *et al.* 2011). This means that some of the adults sampled may belong to neighbouring population and could for some reason, e.g. smell different and therefore not be as reproductively attractive or they could have spawned before migrating up Søre Skotte, and not be contributing to the new offspring.

The relatively short spawning period of grayling in Norway and the fact that they are timing the onset of spawning with the ice break, could be the reason that timing of spawning did not explain the variance in reproductive success in this study. If many grayling are present in the stream from the start of the spawning season there will be nothing to gain with an early-approach strategy.

Conclusion

To my knowledge, I have presented the first results of a genetic study of the grayling breeding system. Its results show grayling of both sexes mating with more than one partner during the 2008 spawning in Lake Lesjaskogsvatnet in Norway. I also observed great variances in the individual reproductive success for both males and females. However, I was not able to explain the observed variances with differences in body length or the timing of spawning run.

Outlook

To investigate a more complete picture of the grayling breeding system it would be necessary to invest in more extensive sampling of adults and offspring, preferentially over multiple spawning seasons, and with respect to the offspring, in smaller time intervals with more overall sampling time points. A more extensive sampling of offspring would also allow for a more satisfying investigation of kinship and cohort composition.

In addition, it might be interesting to investigate several factors that could contribute to the variation in reproductive success in more detail. Body length, which was used here, is only one example and measurement that can be assessed with respect to its carriers' capability to hold a territory as well as its attractiveness for females, but there are many more. To

address the latter, it might be interesting to assess colour intensity and the size of the in males very prominent dorsal fin. In addition, a comparison between those results gained from a genetic investigation of a natural spawning population with data gained through laboratory mate choice experiments under standard conditions would be very interesting.

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